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STUDIES IN EPIDEMIC POLIOMYELITIS

III. COMPARATIVE STUDIES OF COCCI ISOLATED FROM POLIOMYELITIS

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Cocci have been isolated repeatedly from poliomyelitic material, many observers in various localities during the past year having described strains they have found. A comparative study of these strains has seemed desirable to determine whether it is one organism or several organisms the different workers have found, and, if several, what points the different strains have in common.

Accordingly we assembled eight strains and studied their behavior when examined by elementary bacteriologic methods, and also by methods aimed to establish their more intimate functional relationships.

Rosenow, Towne and Wheeler[1] supplied us with 2 strains: No. 44, "the fourth subculture of a strain from a monkey having poliomyelitis following injection of a filtrate of a virus of a monkey that was paralyzed with a filtrate of a virus," and No. 707, "a strain isolated from a single colony from the spinal cord of a case of poliomyelitis in man . . . now in the ninth subculture."

Mathers[2] furnished us with two: Nos. 1 and 7, both from human poliomyelitic material.

Nuzum and Herzog[3] sent us two: No. 1, "from the spinal fluid of a monkey," and No. 2, "from human spinal fluid."

From Dixon and Rucker[4] of Philadelphia we obtained one: No. M 61, from human poliomyelitic material.

From Kolmer, Brown and Freese[5] we obtained one : No. 20, isolated from the pons of a patient who died of poliomyelitis on the seventh day.

Cocci that we have proved to be identical with Streptococcus 20 we have ourselves isolated in the course of cultural work. Glycerinated poliomyelitic nervous tissue from two human cases was emulsified, and one emulsion passed through a Chamberland and the other through a Reichel filter. Cultures from both filtrates gave No. 20. We have, in association with Kolmer, described elsewhere the finding of the globoid bodies of Flexner in these two lots of poliomyelitic material. In another instance, portions of the brain and cord of a

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[1] Jour. Am. Med. Assn., 1916, 67, p. 1202.

[2] Jour. Infect. Dis., 1917, 20, p. 113.

[3] Jour. Am. Med. Assn., 1916, 69, p. 1205.

[4] Report of Commissioner of Health, State of Penna., 1907, p. 434.

[5] Jour. Exper. Med., 1917, 25, p. 789.

monkey with typical anterior poliomyelitis following intracerebral injection of a human virus were distributed among 20 tubes of kidney-acetic fluid medium overlaid with oil. In 9 tubes we found pure cultures of the coccus identical with Streptococcus 20. From the brain of this monkey we were able also to isolate Flexner's globoid bodies.

All 8 strains grow well in air. On glucose-agar slants made from infusion broth, Streptococcus 20, Nuzum's No. 1, and No. M 61 grew abundantly, forming a heavy, smooth, white growth. The other 5 strains gave the fine, discrete, yellowish white growth which is usually associated with the pathogenic streptococci.

When stained smears from broth cultures are examined no constant differences can be detected among the 8 strains. They are all gram-positive and appear singly, as diplococci, in short chains and in small groups. They are of much the same size and no distinct capsule can be made out. All the strains are nonmotile.

When broth cultures are eamined in a hanging drop the 8 strains, according to Chester's[6] classification, fall into 2 groups. No. M 61 and Nuzum's No. 1 show division in more than one direction of space, the cocci appear singly, in pairs, in threes, and in small clusters. The other 6 strains show division in one direction of space only and appear singly, in pairs, and in short chains, but never in threes or in groups, and would be classed with the streptococci. Further investigations were confined to the latter group which showed division in 1 direction of space only, No. M 61 and Nuzum's No. 1 being eliminated.

To determine what action these streptococci have on red blood corpuscles the method described by Lyall[7] was followed. One c.c. of a 2½% suspension of washed sheep corpuscles and 2 c.c. of normal salt solution were placed in test tubes and 0.5 c.c. of 24-hour broth cultures of the streptococci added. The tubes were shaken, placed in a water-bath at 27 C. for 1 hour, then placed in the refrigerator over night. By this method hemolysis of the sheep cells, the production of methemoglobin, or the absence of any action of the bacteria on the corpuscles can be observed.

Strains 1 (Mather's), 7, and 44 were actively hemolytic; Strain 2 gave the brown color of methemoglobin; Strains 20 and 707 had no action on the blood cells.

The action of the 6 strains on inulin, raffinose and salicin was tested. The medium used was sheep-serum-water containing litmus as an indicator, to which was added 1% of the carbohydrate tested. Twenty-four-hour broth cultures of each of the 6 strains were planted in the tubes with a pipet. The tubes were observed for 5 days. Inulin was not attacked by any strain. No. 44 fermented both raffinose and salicin. Nos. 707, 2, and 20 fermented salicin only, while Mather's strains (Nos. 1 and 7) did not produce acid with any one of the 3 carbohydrates.

The fact that none of these strains fermented inulin in our hands, that they are all short chain streptococci, and that none of them produce hydrogen sulphide, would serve to exclude them from any of the Andrewes and Horder's[8] types except Streptococcus mitis or Streptococcus salivarius, the common habitat of both of which is the human mouth.

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[6] A Manual of Determinative Bacteriology, 1914.

[7] Jour. Med. Research, 1914, 30, p. 487.

[8] Systematic Relationships of the Coccaceae, 1908.

As Wright[9] has pointed out, in the serum of animals that have been artificially immunized against a given organism there exist opsonins which resist the heating of the serum to 55 or 60 C. for 10 minutes. We accordingly determined to see if an investigation of the poliomyelitic streptococci along this line would bring out any information of value. To this end we immunized 3 rabbits, each with one of 3 strains of the poliomyelitic streptococci, and tested the opsonizing power of the heated serum of each rabbit against the other strains.

In performing the opsonic test we followed strictly the technic of Wright.[10] The serums were tested within 24 hours after being drawn and were heated in a water-bath at 50 C. for 10 minutes. This amount of heating of normal serum leaves just sufficient opsonin to cause an average intake of less than one coccus per leukocyte. A suspension of cocci containing from 2-4 billion per c.c. is of a satisfactory concentration. When a heavier suspension of organisms is used it is necessary to heat to a higher degree to achieve the desired result of less than one coccus per leukocyte. We have found that a careful adjustment must be made between the amount of heating and the number of organisms in the suspension in order to secure clear-cut results. As a preliminary to each set of tests it is necessary to make a "trial-trip," using heated normal serum, and see that just sufficient phagocytosis remains to be assured that any increased opsonin in the serum will not be influenced by the heating.

TABLE 1

OPSONIC ACTION OF ANTIPNEUMOCOCCIC HORSE SERUM ON TYPES 1, 2, AND 3 OF PNEUMOCOCCI

Serum Heated	Average per Leukocyte of Type 1 Pneumococci	Average per Leukocyte of Type 2 Pneumococci	Average per Leukocyte of Type 3 Pneumococci
Type 1 Serum	5.0	0.0	0.5
Type 2 Serum	0.0	5.0	0.3

In order to see with what delicacy we might expect distinctions to be drawn by this method, we made some preliminary experiments with Types 1, 2 and 3 of pneumococci, and the serum of horses that had been highly immunized against Types 1 and 2. The laboratory of the Bureau of Health of Philadelphia furnished us with the 3 types of pneumococci and with fresh serum. Each serum was tested against the 3 types and increased opsonin was found present only in the serum for the type with which the animal had been injected.

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[9] Studies on Immunization, 1909.

[10] Technique of the Teat and Capillary Glass Tube, 1912.

In view of this result, we felt that if the serum of an animal that had been immunized with one poliomyelitic streptococcus contained increased opsonin for another strain, the 2 strains might rightly be considered as more closely related to each other than are the 3 types of pneumococci. Even should no phagocytosis follow such cross-testing we would still not be justified in denying a close relationship. In the absence of any classification of streptococci to which we can refer for final decision, our results must be stated in relative terms, namely, that 2 strains are more closely related than are the different types of pneumococci or that they are less closely related.

Three rabbits were injected intravenously with living cultures, one with No. 20, one with No. 44, and one with No. 707. Three large injections were given, and, after allowing time for recovery from the negative phase, the serum of each rabbit was tested against the strain with which it had been immunized. With unheated serum such numbers of cocci were taken up that often a count was impossible. When serum heated to 55 C. for 10 minutes was employed, sufficient thermostabile opsonin remained to allow an active phagocytosis.

TABLE 2

OPSONIC ACTION OF UNHEATED AND HEATED SERUM OF IMMUNIZED RABBITS ON THE STRAIN OF POLIOMYELITIC STREPTOCOCCI WITH WHICH THEY WERE INJECTED

	Serum 20 Plus Streptococcus 20	Serum 44 Plus Streptococcus 44	Serum 707 Plus Streptococcus 707
Unheated....................	Uncountable *	17	46
Heated 55 C. for 10 Minutes	16	5	15

* Average per leukocyte.

Assured that each of the serums contained thermostabile opsonin for the strain with which the rabbit had been injected, we proceeded to test each serum against suspensions of the 6 strains of streptococci. In each case the suspension was controlled by using normal rabbit serum heated to 55 C. for 10 minutes.

Examining the results of the tests in which the serum of the rabbit immunized with Streptococcus 20 was used, we see that Strains 20, 1, 7, and 2 were all phagocytized readily, after having been acted on by this immune serum. Evidently these 4 strains are the same so far as this method of investigation is concerned. With Strains 44 and 707 phagocytosis did not occur.

When the serum of the rabbit immunized with Strain 44 was used, phagocytosis took place with Strains 44 and with 707, but not with any of the other strains. It was observed in working with Strain 44 that when the cocci had been acted on by serum they very often became greatly swollen and stained very deeply. This did not occur with any culture except No. 44.

Serum from the rabbit immunized with Strain 707 causes phagocytosis of Strains 707 and 2, but not of any other strain.

We may conclude from this series of tests that Strains 20, 1, 7, and 2 are identical or are more closely related than are the 3 types of pneumococci. The results here are clear-cut and unambiguous. With Strain 707 we are left in some doubt as to the relation it bears to Strains 20, 1, 7, and 2. Possibly it should be grouped with them. Strain 44 would seem by this method of classification to be placed in a separate group from Mather's, Nuzum's and Kolmer's strains.

TABLE 3

OPSONIC ACTION OF IMMUNE RABBIT SERUM ON SIX STRAINS OF POLIOMYELITIC STREPTOCOCCI FROM VARIOUS LOCALITIES

Suspension of Streptococcus	Serum Heated to 55 C. for 10 Minutes			
	Serum of Rabbit Immunized with No. 20	Serum of Rabbit Immunized with No. 44	Serum of Rabbit Immunized with No. 707	Serum of Normal Rabbit
20	16.0 *	0.7	0.7	0.3
44	0.5	5.0	0.7	0.9
707	0.7	2.0	15.0	0.6
1	3.0	0.6	0.9	0.3
7	9.0	0.1	0.7	0.6
2	10.0	0.0	13.0	0.3

* Average per leukocyte.

Agglutination with the coccaceae is admittedly unsatisfactory. The production of agglutinins by the injection of cocci is slow and the agglutinative titer is not high. However, in the endeavor to make our examination of the poliomyelitic cocci as complete as possible, we made a series of agglutination tests with immune rabbit serum, testing each serum with each of the 6 strains of streptococci. The mixtures of serum, normal salt solution and cocci were made and incubated in capillary glass tubes according to the method described by Wright.[10] The results were read after the mixtures had been incubated 24 hours.

The results of the agglutinations parallel closely the results of the opsonic tests, but the distinctions drawn are less fine. The agglutination method detects no difference whatever between Strains 20, 1, 7, 2, and 707. By the opsonic method, it will be remembered, the identity of Strain 707 with the other 4 was uncertain. In the agglutinations, Strain 44 again stands out as a strain of another type.

TABLE 4

AGGLUTINATION OF SIX STRAINS OF POLIOMYELITIC STREPTOCOCCI BY THE SERUMS OF RABBITS IMMUNIZED WITH DIFFERENT STRAINS

	Dilutions				
	1:4	1:8	1:16	1:32	Control
Serum of Rabbit Immunized with Strain 20 plus					
Suspension of No. 20	+	+	+	+	—
Suspension of No. 44	—	—	—	—	—
Suspension of No. 707	+	+	+	+	—
Suspension of No. 1	+	+	+	+	—
Suspension of No. 7	+	+	+	—	—
Suspension of No. 2	+	+	+	+	—
Serum of Rabbit Immunized with Strain 44 plus					
Suspension of No. 20	+	±	—	—	—
Suspension of No. 44	+	—	—	—	—
Suspension of No. 707	+	—	—	—	—
Suspension of No. 1	—	—	—	—	—
Suspension of No. 7	—	—	—	—	—
Suspension of No. 2	±	—	—	—	—
Serum of Rabbit Immunized with Strain 707 plus					
Suspension of No. 20	+	+	+	—	—
Suspension of No. 44	—	—	—	—	—
Suspension of No. 707	+	+	—	—	—
Suspension of No. 1	+	—	+	—	—
Suspension of No. 7	+	—	—	—	—
Suspension of No. 2	+	+	+	+	—
Serum of Normal Rabbit plus					
Suspension of No. 20	±	—	—	—	—
Suspension of No. 44	—	—	—	—	—
Suspension of No. 707	±	—	—	—	—
Suspension of No. 1	—	—	—	—	—
Suspension of No. 7	—	—	—	—	—
Suspension of No. 2	—	—	—	—	—

SUMMARY

With the object of determining whether it is one strain of streptococci so commonly found associated with poliomyelitis, or several, we secured cultures from investigators who have isolated strains from poliomyelitis material in various parts of the country. We have grown them side by side on the same mediums. We have applied to them fermentative and other cultural tests found useful in the classification of streptococci. We have immunized rabbits with strains from different sources and with the immune serum so obtained have made opsonic tests on each strain to see if the heated serum giving phagocytosis with the strain with which the rabbit was injected will also cause phagocytosis of the other strains. With these same immune rabbit serums we also made cross agglutination tests.

The six strains of streptococci examined by cultural methods appear to be identical or closely related.

When examined by methods tending to bring out their functional activities Kolmer's strain, Mather's strains, and Nuzum's strain from human spinal fluid are identical. Rosenow's Strain 44 exhibits differences from the first group which are at least as great as the differences between Types 1 and 2 of pneumococci. Rosenow's Strain 707 gives limited reactions with some members of both of the 2 former groups.

While under strict anaerobic conditions the prolonged cultivation of streptococci results in a diminution of size and a tendency to become gram-negative, yet the biologic and morphologic differences between the globoid bodies and the small form of streptococci appear to be distinct.